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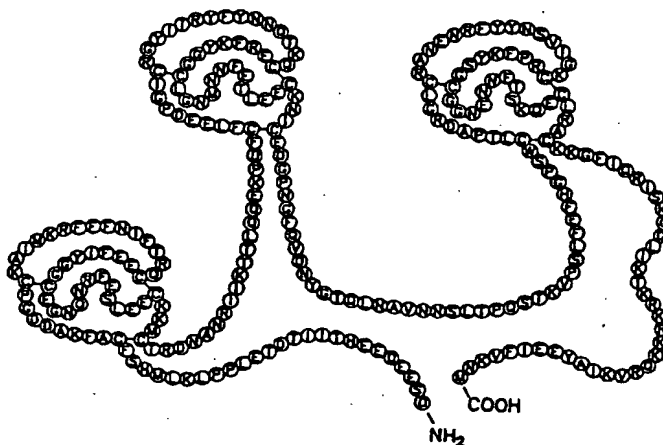
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(54) Title: ANTICOAGULANT PROTEIN



(57) Abstract

Novel EPI analogues are provided wherein one or more of the amino acid residues of native EPI have been deleted. A preferred group of the novel EPI analogues have no or a low heparin binding capacity. The novel EPI analogues can be used for the treatment of patients having coagulation disorders or cancer.

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ANTICOAGULANT PROTEINField of the invention

The present invention relates to a new protein with EPI activity, a method of producing such protein and a therapeutic preparation containing such protein.

Background of the invention

Blood coagulation is a complex process involving many activating and inactivating coagulation factors. Anticoagulant proteins are known to be important for regulation of the coagulation process (see B. Lämmle and J. Griffin (Clinics in Haematology 14 (1985), 281-342) and anticoagulants are thus important in the treatment of a variety of diseases, eg thrombosis, myocardial infarction, disseminated intravascular coagulation etc.

Thus heparin is used clinically to increase the activity of antithrombin III and heparin cofactor II. Antithrombin III is used for the inhibition of factor Xa and thrombin. Hirudin is used for the inhibition of thrombin and protein C may be used for the inhibition of factor V and factor VIII.

Anticoagulant proteins may also be used in the treatment of cancer. Thus, antistatin has been shown to have anti-metastatic properties (J.H. Han et al., Gene 75 (1989), 47-57). Also heparin and warfarin have been shown to possess antimetastatic properties (G.J. Gasic et al., Int. Rev. Exp. Pathol. 29 (1985), 173-209).

Coagulation can be initiated through the extrinsic pathway by the exposure of tissue factor (TF) to the circulating blood (Y. Nemerson, Blood 71 (1988), 1-8). Tissue factor is a protein cofactor for factor VII/VIIa and binding of tissue factor enhances the enzymatic activity of factor VIIa (FVIIa) towards

its substrates factor IX and factor X. Placenta anticoagulant protein is able to inhibit tissue factor activity, probably by interfering with TF/FVIIa-phospholipid interaction (S. Kondo et al., *Thromb. Res.* 48 (1987), 449-459).

5 Recently a new anticoagulant protein, the extrinsic pathway inhibitor (EPI) has been isolated (Broze et al., *Proc. Natl. Acad. Sci.* 84 (1987), 1886-1890).

On a molar basis EPI has been shown to be a potent inhibitor of TF/FVIIa induced coagulation (R.A. Gramzinski et al., *Blood* 73
10 (1989), 983-989). EPI binds and inhibits factor Xa (FXa) and the complex between EPI and FXa inhibits TF/FVIIa (Rapaport, *Blood* 73 (1989), 359-365). EPI is especially interesting as an anticoagulant/antimetastatic agent as many tumor cells express TF activity (T. Sakai et al., *J. Biol. Chem.* 264 (1989), 9980-
15 9988) and because EPI shows anti-Xa activity like antistatin.

EPI has been recovered by Broze et al. (supra) from HepG2 hepatoma cells (Broze EP A 300988) and the gene for the protein has been cloned (Broze EP A 318451). A schematic diagram over the secondary structure of EPI is shown in Fig. 1 and the amino
20 acid sequence of EPI is shown in Fig. 2 where the N-terminal amino acid Asp is given the number 1. The protein consists of 276 amino acid residues and has in addition to three inhibitor domains of the Kunitz type three potential glycosylation sites at position Asn117, Asn167 and Asn229. The molecular weight
25 shows that some of these sites are glycosylated. Furthermore, it has been shown that the second Kunitz domain binds FXa while the first Kunitz domain binds FVIIa/TF (Girard et al., *Nature* 338 (1989), 518-520). EPI has also been isolated from HeLa cells (PCT/DK90/00016) and it was shown that HeLa EPI binds
30 heparin.

Heparin binding is an important factor for the pharmacokinetics of substances for injection. It has been shown that platelet factor 4 (M. Prosdomi et al., *Thromb. Res.* 39 (1985), 541-

547) and aprotinin with one Kunitz domain (H. Fritz et al., Drug Res. 33, 479-94) has a short half life probably due to the heparin binding properties. Lowering of the heparin binding capacity of an anticoagulant would therefore seem to be 5 advantageous. Furthermore, it might be advantageous to use a smaller molecule than EPI for the medical treatment.

It has according to the present invention surprisingly been found that certain EPI analogues retain the EPI activity as well as anti Xa activity although parts of the molecule has 10 been deleted. Furthermore, these analogues show a much lower affinity for heparin than full length EPI, making them more useful as therapeutic agents than the native molecule. The EPI analogues will furthermore have a longer half life as compared with native EPI which will further reduce the amount of active 15 ingredients for the medical treatment.

Summary of the invention

In its first aspect the present invention is related to novel EPI analogues wherein one or more of the amino acid residues of native EPI have been deleted.

20 In its second aspect the present invention is related to a new group of EPI analogues having EPI activity but with no or low heparin binding capacity under physiological conditions (pH, ionic strength).

In the present context the term "low heparin binding capacity" 25 is intended to mean a binding capacity of about 50%, more preferably of about 25% and most preferably less than about 10% of that of native EPI at physiological pH and ionic strength.

The preferred group of the novel EPI analogues can be characterized as being devoid of the heparin binding domain of native EPI or having a non-functional heparin binding domain by 30 having deleted one or more of the amino acid residues in said

domain resulting in loss or a substantial lowering of the heparin binding capacity. The same effect may also be obtained by substituting one or more of the amino acid residues in the heparin binding domain with another amino acid residue.

5 Detailed description of the invention

To retain the EPI activity the analogues according to the present invention should at least contain the N-terminal sequence including the first and second Kunitz domain. Thus, the EPI analogues according to the present invention should at least contain the amino acid sequence from amino acid number 25 to amino acid number 148 of native EPI (see fig. 1 and 2).

It has been shown by the inventors hereof that the heparin binding capacity is lost when the sequence from amino acid residue number 162 to amino acid residue number 275 is deleted from the EPI molecule. It is therefore concluded that the heparin binding domain is situated in this part of the EPI molecule. It is assumed that the heparin binding domain comprises at least a region from Arg246 to Lys265 near the C-terminal end of the EPI molecule being rich in positively charged amino acid residues.

Preferred EPI analogues according to the present invention are such in which one or more amino acid residues have been deleted in the native EPI molecule from amino acid residue Glu148 to the C-terminal Met276.

More specifically, one or more amino acid residues in the sequence from Arg246 to Lys275 have been deleted.

Examples of EPI analogues according to the present invention are:

5
10
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25
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(Asp1 - Thr255)-EPI
(Asp1 - Ile253)-EPI
(Asp1 - Lys249)-EPI
(Asp1 - Ser248)-EPI
(Asp1 - Lys240)-EPI
(Asp1 - Glu234)-EPI
(Asp1 - Trp188)-EPI
(Asp1 - Asn164)-EPI
(Asp1 - Thr161)-EPI
(Asp1 - Asp149)-EPI
(Asp1 - Glu148)-EPI

Ser-(Asp1 - Thr255)-EPI
Ser-(Asp1 - Ile253)-EPI
Ser-(Asp1 - Lys249)-EPI
Ser-(Asp1 - Ser248)-EPI
Ser-(Asp1 - Lys240)-EPI
Ser-(Asp1 - Glu234)-EPI
Ser-(Asp1 - Trp188)-EPI
Ser-(Asp1 - Asn164)-EPI
Ser-(Asp1 - Thr161)-EPI
Ser-(Asp1 - Asp149)-EPI
Ser-(Asp1 - Glu148)-EPI

(Glu15 - Thr255)-EPI
(Glu15 - Ile253)-EPI
(Glu15 - Lys249)-EPI
(Glu15 - Ser248)-EPI
(Glu15 - Lys240)-EPI
(Glu15 - Glu234)-EPI
(Glu15 - Trp188)-EPI
(Glu15 - Asn164)-EPI
(Glu15 - Thr161)-EPI
(Glu15 - Asp149)-EPI
(Glu15 - Glu148)-EPI

Ser-(Glu15 - Thr255)-EPI
Ser-(Glu15 - Ile253)-EPI
Ser-(Glu15 - Lys249)-EPI
Ser-(Glu15 - Ser248)-EPI
5 Ser-(Glu15 - Lys240)-EPI
Ser-(Glu15 - Glu234)-EPI
Ser-(Glu15 - Trp188)-EPI
Ser-(Glu15 - Asn164)-EPI
Ser-(Glu15 - Thr161)-EPI
10 Ser-(Glu15 - Asp149)-EPI
Ser-(Glu15 - Glu148)-EPI

(Ser24 - Thr255)-EPI
(Ser24 - Ile253)-EPI
(Ser24 - Lys249)-EPI
15 (Ser24 - Ser248)-EPI
(Ser24 - Lys240)-EPI
(Ser24 - Glu234)-EPI
(Ser24 - Trp188)-EPI
(Ser24 - Asn164)-EPI
20 (Ser24 - Thr161)-EPI
(Ser24 - Asp149)-EPI
(Ser24 - Glu148)-EPI

(Asp1 - Thr255)-(Ile266 - Met276)-EPI
(Asp1 - Ile253)-(Ile266 - Met276)-EPI
25 (Asp1 - Ser248)-(Ile266 - Met276)-EPI
(Asp1 - Gln245)-(Ile266 - Met276)-EPI
(Asp1 - Thr255)-(Val264 - Met276)-EPI
(Asp1 - Ile253)-(Val264 - Met276)-EPI
(Asp1 - Ser248)-(Val264 - Met276)-EPI
30 (Asp1 - Glu245)-(Val264 - Met276)-EPI
(Asp1 - Thr255)-(Glu262 - Met276)-EPI
(Asp1 - Ile253)-(Glu262 - Met276)-EPI
(Asp1 - Ser248)-(Glu262 - Met276)-EPI
(Asp1 - Glu245)-(Glu262 - Met276)-EPI

Ser-(Asp1 - Thr255)-(Ile266 - Met276)-EPI
 Ser-(Asp1 - Ile253)-(Ile266 - Met276)-EPI
 Ser-(Asp1 - Ser248)-(Ile266 - Met276)-EPI
 Ser-(Asp1 - Gln245)-(Ile266 - Met276)-EPI
 5 Ser-(Asp1 - Thr255)-(Val264 - Met276)-EPI
 Ser-(Asp1 - Ile253)-(Val264 - Met276)-EPI
 Ser-(Asp1 - Ser248)-(Val264 - Met276)-EPI
 Ser-(Asp1 - Glu245)-(Val264 - Met276)-EPI
 Ser-(Asp1 - Thr255)-(Glu262 - Met276)-EPI
 10 Ser-(Asp1 - Ile253)-(Glu262 - Met276)-EPI
 Ser-(Asp1 - Ser248)-(Glu262 - Met276)-EPI
 Ser-(Asp1 - Glu245)-(Glu262 - Met276)-EPI

In addition to the described deletions in the EPI molecule, certain amino acid residues of native EPI may also be replaced
 15 by another naturally occurring amino acid residue. The EPI analogues may also advantageously contain a Ser residue as the N-terminal residue. This is necessary if a signal sequence is used requiring an N-terminal Ser in the mature protein as a recognition site for cleavage. Thus, the N-terminal in the EPI
 20 molecule may be replaced by a Ser or an additional Ser may be inserted adjacent to the original N-terminal residue. Also the potential glycosylation sites at Asn167 and Asn238 may be substituted by another amino acid residue to avoid glycosylation.

25 It has not previously been shown that glycosylation sites are dispensable for EPI activity. Neither has it been shown that large fragments of the EPI protein are dispensable for EPI activity, i.e. for FXa dependent inhibition of TF/FVIIa. It has previously been shown that a single amino acid in EPI can be
 30 replaced by another amino acid residue (Arg199 -> Leu199), (Girard et al., Nature 338 (1989), 518-520) without affecting the activity. However, such a change does not significantly change the structure of the protein. In contrast hereto the deletions according to the present invention give rise to new

molecules with other properties than the native molecule, as illustrated by the changed affinity for heparin.

The present EPI-analogues may be produced by well-known recombinant DNA technology.

5 The gene for the native EPI has been cloned and sequenced (Wun et al., J. Biol. Chem. 263 (1988), 6001-6004). DNA-sequences encoding the desired EPI-analogue may be constructed by altering EPI cDNA by site-directed mutagenesis using synthetic oligonucleotides encoding the desired amino acid sequence for
10 homologous recombination in accordance with well-known procedures.

The DNA sequence encoding the EPI analogue of the invention may also be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by S.L. Beaucage and
15 M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoramidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable
20 vectors.

In a further aspect, the present invention relates to a recombinant expression vector which comprises a DNA sequence encoding the EPI analogue of the invention. The expression vector may be any vector which may conveniently be subjected to
25 recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication,
30 e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genom and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the EPI analogue of the invention should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the EPI analogue of the invention in mammalian cells are the SV 40 promoter (Subramani et al., Mol. Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814), the adenovirus 2 major late promoter or the CMV (cytomegalovirus IE1) promoter (Henninghausen et al., EMBO J. 5 (1986), 1367-71). Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al., eds.), Plenum Press, New York, 1982), or the PTI1 (US 4,599,311) or ADH2-4c (Russell et al., Nature 304, 1983, pp. 652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) or the tipA promoter.

To ensure secretion a suitable signal sequence is inserted at the 5' of the DNA sequence encoding the EPI analogue. A suitable signal sequence is the t-PA signal sequence (Friezner et al., J. Biol. Chem. 261 (1986), 6972-85).

The DNA sequence encoding the EPI analogue of the invention should also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) promoters. The vector may further comprise elements such as polyadenylation

signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

- 5 The recombinant expression vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication. The vector may also comprise a selectable marker,
10 e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g. neomycin, hygromycin or methotrexate.

The procedures used to ligate the DNA sequences coding for the
15 EPI analogue of the invention, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op. cit.).

- 20 In a further aspect, the present invention relates to a cell which contains the recombinant expression vector described above. The host cell may be any cell which is capable of producing the EPI analogue and is preferably a eukaryotic cell, in particular a mammalian cell. Examples of suitable mammalian
25 cell lines are the COS (ATCC CRL 1650 and 1651), BHK (ATCC CRL 1632, ATCC CCL 10) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J.Mol.Biol. 159, 1982, pp. 601-621; Southern and Berg,
30 J.Mol.Appl.Genet. 1, 1982, pp. 327-341; Loyter et al., Proc.Natl.Acad.Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and Pearson, Somatic Cell Genetics 7, 1981, p. 603, Graham and van der Eb, Virology 52, 1973, p. 456; and Neuman et al., EMBO J. 1, 1982, pp. 841-845.

Alternatively, fungal cells (including yeast cells) may be used as host cells of the invention. Examples of suitable yeast cells include cells of Saccharomyces spp. or Schizosaccharomyces spp., in particular strains of 5 Saccharomyces cerevisiae. Examples of other fungal cells are cells of filamentous fungi, e.g. Aspergillus spp. or Neurospora spp., in particular strains of Aspergillus oryzae or Aspergillus niger. The use of Aspergillus spp. for the expression of proteins is described in, e.g., EP 272,277.

10 In a still further aspect the present invention relates to a process for producing an EPI analogue according to the invention, which comprises culturing a cell as described above in a suitable nutrient medium under conditions which are conductive to the expression of the EPI analogue, and 15 recovering the polypeptide from the culture. The medium used to culture the cells may be any conventional medium suitable for growing mammalian cells, such as a serum-containing or serum-free medium containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared 20 according to published recipes (e.g. in catalogues of the American Type Culture Collection).

The EPI analogue will preferably be secreted to the growth medium and may be recovered from the medium by conventional procedures including separating the host cells from the medium 25 by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the 30 like.

The novel EPI analogues may be used for the treatment of patients having coagulation disorders or cancer.

Accordingly the invention is also related to a pharmaceutical preparation for the treatment of patients having coagulation disorders or cancer containing an EPI analogue in a suitable amount together with suitable adjuvants and additions.

5 The pharmaceutical preparations may be in a buffered aqueous solution with appropriate stabilizers and preservatives. The solution may be heat treated and may be contained in ampoules or in carpoules for injection pens. Alternatively the stabilized solution may be freeze dried and contained in
10 ampoules or in two chamber injection systems with freeze dried substance in one chamber and solvent in the other chamber.

Brief description of the Drawings

The present invention is further described with reference to the drawings in which

- 15 Fig. 1 shows the amino acid sequence and the two dimensional structure of EPI,
- Fig. 2 shows the amino acid sequence of native EPI,
- Fig. 3 shows plasmid pJR77 containing the t-PA signal, the human cytomegalovirus IE1 gene promoter and the human
20 growth hormone gene polyadenylation signal,
- Fig. 4 shows pCMVEPIhGH containing the EPI gene,
- Fig. 5 shows plasmid pEP1b1 containing a DNA sequence encoding Ser-(Thr88 - Thr161)-EPI,
- Fig. 6 shows plasmid PEPlab containing a DNA sequence
25 encoding Ser-(Glu15 - Thr161)-EPI,
- Fig. 7 shows the DNA sequence for Ser-(Thr88 - Thr161)-EPI preceded by the t-PA signal sequence,

Fig. 8 shows the DNA-sequenc for the Ser-(Glu15 - Thr161)-EPI preceded by the t-PA signal sequence,

Fig. 9 shows plasmid PEPIab2 containing a DNA sequence encoding (Asp1 - Thr161)-EPI, and

5 Fig. 10 shows the DNA sequence for (Asp1 - Thr161)-EPI preceded by the EPI signal.

The invention is further described in the following examples which are not in any ways intended to limit the scope or spirit of the invention as claimed.

10 Experimental Part

Assay for EPI activity: EPI was measured in a chromogenic microplate assay, modified after the method of Sandset et al., (Thromb. Res. 47 (1989), 389-400). Heat treated plasma pool was used as a standard. This standard is set to contain 1 U/ml
15 of EPI activity. Standards and samples were diluted in buffer A (0.05 M tris / 0.1 M NaCl / 0.1 M Na-citrate / 0.02% NaN₃ / pH 8.0) containing 2 µg/ml polybrene and 0.2% bovine serum albumin. FVIIa/TF/FX/CaCl₂ combination reagent was prepared in buffer A and contained 1.6 ng/ml FVIIa (Novo-Nordisk a/s),
20 human tissue factor diluted 60 fold (Hjort, Scand. J. Clin. Lab. Invest. 2 (1957), 50 ng/ml FX (Sigma) and 18 mM CaCl₂. The assay was performed in microplate strips at 37°C. 50 µl of samples and standards were pipetted into the strips and 100 µl combination reagent was added to each well. After 10 minutes
25 incubation, 25 µl of FX (3.2 µg/ml) was added to each well and after another 10 minutes 25 µl of chromogenic substrate for FXa (S2222) was added 10 minutes after the addition of substrate. The reaction was stopped by addition of 50 µl 1.0 M citric acid pH 3.0. The microplate was read at 405 nm.

Assay for anti Xa activity: HeLa EPI purified on heparin sepharose (PCT/DK90/00016) was used as a standard. This standard was assigned an amount of Xa inhibition units corresponding to the amount of EPI units measured in the EPI assay. Samples and 5 standards were diluted in 50 mM tris/0.2% bovine serum albumin pH 7.3. 100 μ l of diluted samples and standards were incubated 30 minutes at 37°C with 100 μ l FXa (Stago, 14 ng/ml). 25 μ l of S2222 (2 mg/ml) was added after another 2 hours at 37°C. The assay was stopped and read like the EPI assay.

10 Synthetic oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystem DNA synthesizer.

M13 sequencing primers and gamma-[³²P]-ATP (5000 Ci/mmol, 10 mCi/ml) for labelling of primers were obtained from Amersham.

Restriction endonucleases and T4 DNA-ligase were obtained from 15 New England Biolabs. Modified T7 DNA-polymerase (Sequenase) was obtained from United States Biochemicals. pBS+ (Stratagene) was used as cloning vector for synthetic DNA fragments.

A mammalian expression vector denoted pJR77 (fig. 3) containing the human cytomegalovirus IE1 gene promoter and the human 20 growth hormone gene polyadenylation signal was used for expression of various EPI related proteins in COS-7 cells.

XL-1 Blue (Stratagene) a derivative of *E. coli* K12 was used as bacterial recipient for plasmid transformations and as host for propagation and preparation of plasmid DNA.

25 Green monkey kidney cell-line COS-7 (ATCC # CRL 1651) was grown in Dulbecco's modified eagle medium (DMEM) (Gibco 041-1965) + 50 μ g/ml gentamycin + 110 μ g/ml pyruvate + 10% fetal calf serum (FCS) or DMEM + 50 μ g/ml gentamycin + 110 μ g/ml pyruvate + 1% ITS* (insulin, transferrin, serum albumin).

Restriction endonucleases and other enzymes were used in accordance with the manufacturers recommendations. Standard recombinant DNA-techniques were carried out as described (Maniatis et al., Molecular cloning. Cold Spring Harbor Laboratory, 1982).

DNA sequences were determined by the dideoxy chain termination method (Sanger et al., Proc. Natl. Acad. Sci. 74 (1977), 5463-67) using double stranded plasmid DNA as template and ³²P-labelled primers and Sequenase.

10 Plasmid DNA was introduced into COS-7 cells by calcium phosphate coprecipitation (Graham & van der Eb, Virology 52 (1973), 456-457).

7x10⁵ cells were seeded in 20 cm² dishes in 5 ml DMEM + FCS. The following day each dish was added 20 µg plasmid DNA in 0.5 precipitate and 50 µl 10 mM chloroquine diphosphate. Cells were incubated overnight with precipitate. The following day fresh medium DMEM + ITS* was added. After two days of incubation media were harvested and assayed for EPI-activity and anti Xa-activity.

20 Example 1

Preparation of DNA-fragments encoding EPI and EPI analogues and transient expression in COS-7 cells.

The EPI cDNA sequence is described by Wun et al. (J. Biol. Chem. 263 (1988), 6001-6004). Based on the known sequence
25 synthetic DNA-sequences encoding EPI and EPI analogues were constructed by silent mutations introducing restriction endonuclease recognition sites at suitable locations.

Expression cassettes encoding EPI fragments using the tPA signal sequence (fig. 7 and 8) and an expression cassette encoding the entire EPI protein were assembled by sequentially
30 insertion into the cloning vector pBS+ of annealed complemen-

tary synthetic oligonucleotides. Following assembly of DNA fragment sequences were verified by DNA sequencing as described.

Finally expression cassettes were transferred to expression vector pJR77 giving rise to the following expression plasmids:

pEPIb1 encoding the tPA signal followed by EPI analogue Ser-(Thr88 - Thr161)-EPI.

pEPIab encoding the tPA signal followed by EPI analogue Ser-(Glu15 - Thr161)-EPI.

10 pCMVEPIhGH encoding the entire EPI protein.

COS-7 cells were transfected as indicated in Table 1. After 2 days of incubation with DMEM + ITS⁺ media were harvested. Table 1 shows assay results for EPI-activity and anti-Xa activity.

Table 1.

| 15 | Plasmid DNA | EPI [U/ml] | anti-Xa [U/ml] |
|----|----------------|---------------|-------------------|
| 20 | pBS+ | 0.19 | 0.19 |
| | pCMVEPIhGH | 8.3 | 7.6 |
| | pEPIb1 | 0.28 | 0.15 |
| | pEPIab | 4.5 | 5.6 |

The results in table 1 show that the EPI-fragment Ser-(Thr88 - Thr161)-EPI containing only the second Kunitz domain has no activity whereas the EPI-fragment Ser-(Glu15 - Thr161)-EPI containing the first and second Kunitz domain has retained the EPI activity.

Example 2

COS transfections with EPI plasmids were performed as described in example 1 and media were harvested after 48 hours. 5 columns were packed, each with 300 ul of heparin sepharose. 5 The columns were equilibrated with 20 mM tris/10% glycerol, pH 7.5. (buffer B). 1.3 ml culture medium was applied to each column. Then the columns were washed with 1.5 ml buffer B and each column was eluted with steps of 1.5 ml buffer B with increasing amounts of NaCl. For comparison is given data from 10 another experiment where EPI from HeLa and HepG2 cells were fractionated on heparin-sepharose. In this experiment other NaCl concentrations were used for elution. Table 2 shows that Ser-(Glu15 - Thr161)-EPI does not bind to heparin at physiological pH and ionic strength.

Table 2. Heparin binding of EPI and EPI-like proteins

| 5 | Culture medium | U EPI in medium | flow through | % of EPI activity | | | | |
|----|--|-----------------|--------------|-------------------|-------------|-----|------|-----|
| | | | | B wash | 0.25 M NaCl | 0.5 | 0.75 | 2.0 |
| 10 | untransfected COS | 0.25 | <4 | <4 | <4 | 17 | 54 | 33 |
| | EPI-transfected COS | 7.10 | <4 | <4 | <4 | 6 | 48 | <4 |
| | Ser-(Glu15-Thr161)-EPI transfected COS | 8.34 | 76 | 24 | <3 | <3 | <3 | <3 |
| 20 | untransfected HeLa | 1.17 | NM | NM | ND | 64 | ND | 15 |
| | untransfected HepG2 | 1.95 | NM | NM | ND | 12 | ND | 74 |

NM: not measured, ND: not done

Example 3Preparation of (Asp1 - Thr161)-EPI

Construction of expression plasmids, transformation and
 30 expression in COS-7 cell was performed using materials and methods as described in Example 1.

Sequences between SalI and BamHI of expression plasmid pEPIab (Fig. 6) encoding the t-PA signal and Ser-(Glu15 - Thr161)-EPI was replaced by a synthetic DNA-sequence encoding the
 35 authentic EPI signal and (Asp1 - Thr161)-EPI.

The resulting plasmid pEPIab2 is shown in Fig. 9 and the expression cassette of pEPIab2 is shown in Fig 10.

Culture medium from pEPIab2 transfected COS cells were applied to Heparin-Sepharose as described in Example 2. 3.8 ml culture medium containing 31.2 U/ml of EPI was applied to a 0.5 ml heparin column. Flow through contained 77% and B wash contained 5 16% of the applied EPI activity. No EPI was detected in eulates with 0.25, 0.75 and 1.5 M NaCl respectively.

(Asp1 - Thr161)-EPI has one potential N-linked glycosylation site (Asn117) and the importance of this glycosylation for activity was investigated.

10 (Asp1 - Thr161)-EPI was purified from COS culture medium by affinity chromatography of FXa-Sepharose. In SDS-PAGE the purified protein appeared as a glycosylated band near 27 kDa and an unglycosylated band near 22 kDa (shown by treatment with endogylcosidase F). The glycosylated and unglycosylated forms
15 were separated in unreduced SDS-PAGE and were extracted from the gel. Both forms were active in the EPI assay and showed the same specific activity as judged from the staining intensities in the SDS-gel. Glycosylation at Asn117 is therefore apparently not essential for EPI activity and active (Asp1 - Thr161)-EPI
20 can thus be obtained in efficient expression systems where mammalian N-linked glycosylation is not obtained, e.g. in procaryots, or as unsecreted protein in yeast.

CLAIMS

1. EPI analogue, characterized in that it has a low heparin binding capacity or does not bind to heparin under physiological conditions.
- 5 2. EPI analogue according to claim 1, wherein the heparin binding domain has been deleted or made non-functional by deleting one or more of the amino acid residues in said domain or substituting one or more of the amino acid residues in said domain with other naturally occurring amino acid residues.
- 10 3. EPI analogue according to claim 1 or 2, characterized in that it at least contains the first and second Kunitz domain of native EPI.
4. EPI analogue according to claim 3, characterized in that it at least contains the amino acid sequence from Phe25 to
15 Glu148 of native EPI.
5. EPI analogue according to one or more of the previous claims, wherein one or more of the amino acid residues in the sequence from Asp149 to the C-terminal Met276 of native EPI have been deleted.
- 20 6. EPI analogue according to claim 5 wherein one or more of the amino acid residues in the sequence from Arg246 to Lys265 of native EPI have been deleted.
7. EPI analogue according to claim 1 comprising the amino acid sequence from Asp1 to Arg249 of native EPI.
- 25 8. EPI analogue according to claim 1 comprising the amino acid sequence from Asp1 to Glu235 of native EPI.
9. EPI analogue according to claim 1 comprising the amino acid sequence from Asp1 to Thr161 of native EPI.

10. EPI analogue according to claim 1 comprising the amino acid sequence from Glu15 to Arg249 of native EPI.
11. EPI analogue according to claim 1 comprising the amino acid sequence from Glu15 to Gln235 of native EPI.
- 5 12. EPI analogue according to claim 1 comprising the amino acid sequence from Glu15 to Thr161 of native EPI.
13. EPI analogue according to any of the previous claims and further having a Ser as the N-terminal amino acid residue.
14. DNA-sequence encoding an EPI analogue according to any of
10 the previous claims.
15. Expression vector containing a DNA-sequence according to claim 14.
16. Transformed or transfected microorganism or cell line comprising a vector according to claim 15.
- 15 17. Method for preparation of EPI analogues according to any of the previous claims wherein a microorganism or cell line according to claim 16 is cultured in a suitable culture medium whereupon the EPI analogue is isolated.
- 20 18. A therapeutic preparation for the treatment of patients having coagulation disorders or cancer, characterized in that it contains an EPI-analogue according to any of the previous claims 1-13 and suitable adjuvants and additions.

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Asp Ser Glu Glu Asp Glu Glu His Thr Ile Ile Thr Asp Thr Glu Leu
 1 5 10 15
 Pro Pro Leu Lys Leu Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp
 20 25 30
 Gly Pro Cys Lys Ala Ile Met Lys Arg Phe Phe Phe Asn Ile Phe Tyr
 35 40 45
 Arg Gln Cys Glu Glu Phe Ile Tyr Gly Gly Cys Glu Gly Asn Gln Asn
 50 55 60
 Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met Cys Thr Arg Asp Asn
 65 70 75 80
 Ala Asn Arg Ile Ile Lys Thr Thr Leu Gln Gln Glu Lys Pro Asp Phe
 85 90 95
 Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys Arg Gly Tyr Ile Thr Arg
 100 105 110
 Tyr Phe Tyr Asn Asn Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly
 115 120 125
 Gly Cys Leu Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys
 130 135 140
 Asn Ile Cys Glu Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr Gly
 145 150 155 160
 Thr Gln Leu Asn Ala Val Asn Asn Ser Leu Thr Pro Gln Ser Thr Lys
 165 170 175
 Val Pro Ser Leu Phe Glu Phe His Gly Pro Ser Trp Cys Leu Thr Pro
 180 185 190
 Ala Asp Arg Gly Leu Cys Arg Ala Asn Glu Asn Arg Phe Tyr Tyr Asn
 195 200 205
 Ser Val Ile Gly Lys Cys Arg Pro Phe Lys Tyr Ser Gly Cys Gly Gly
 210 215 220
 Asn Glu Asn Asn Phe Thr Ser Lys Gln Glu Cys Leu Arg Ala Cys Lys
 225 230 235 240
 Lys Gly Phe Ile Gln Arg Ile Ser Lys Gly Gly Leu Ile Lys Thr Lys
 245 250 255
 Arg Lys Arg Lys Lys Gln Arg Val Lys Ile Ala Tyr Glu Glu Ile Phe
 260 265 270
 Val Lys Asn Met
 275

Fig. 2

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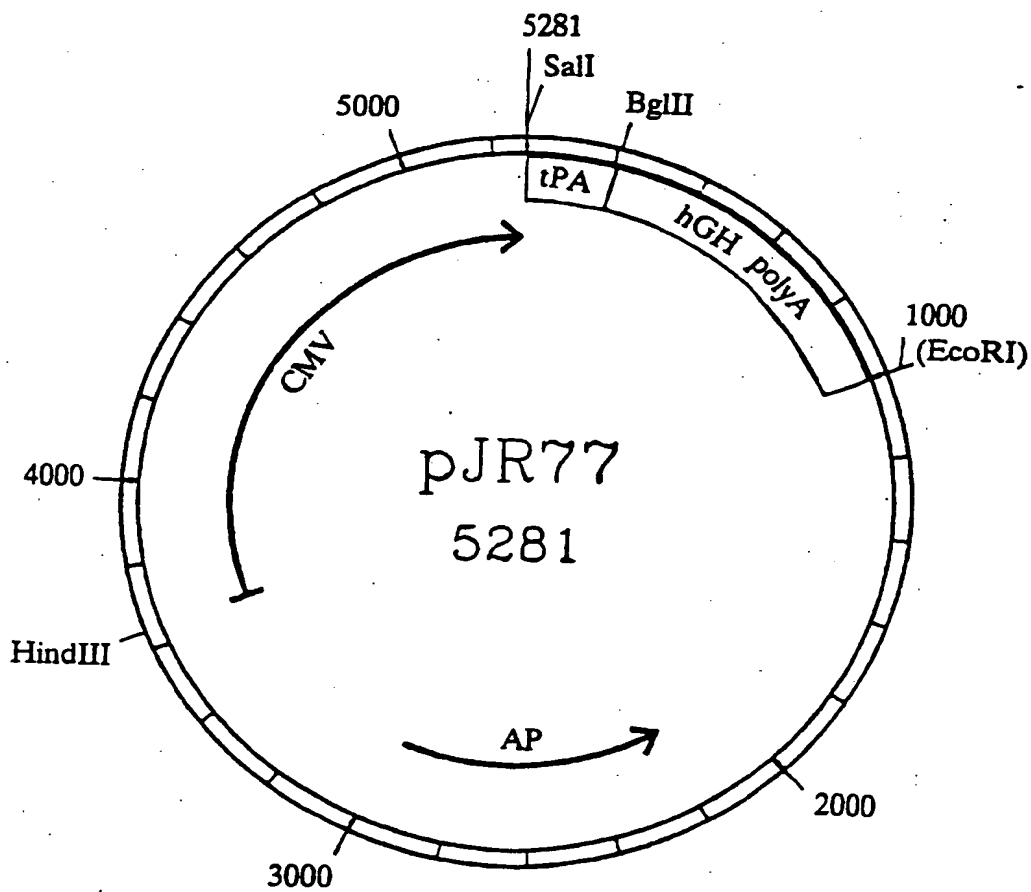


Fig. 3

REPLACEMENT SHEET

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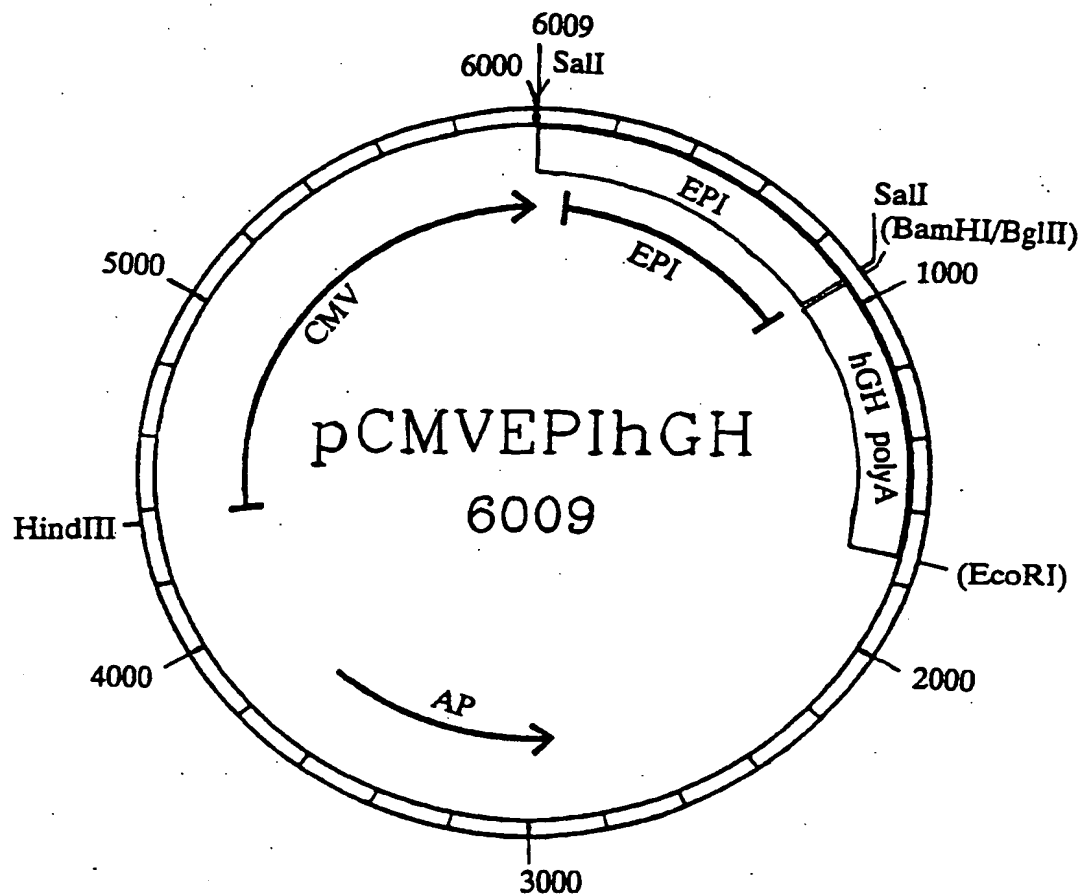


Fig. 4

REPLACEMENT SHEET

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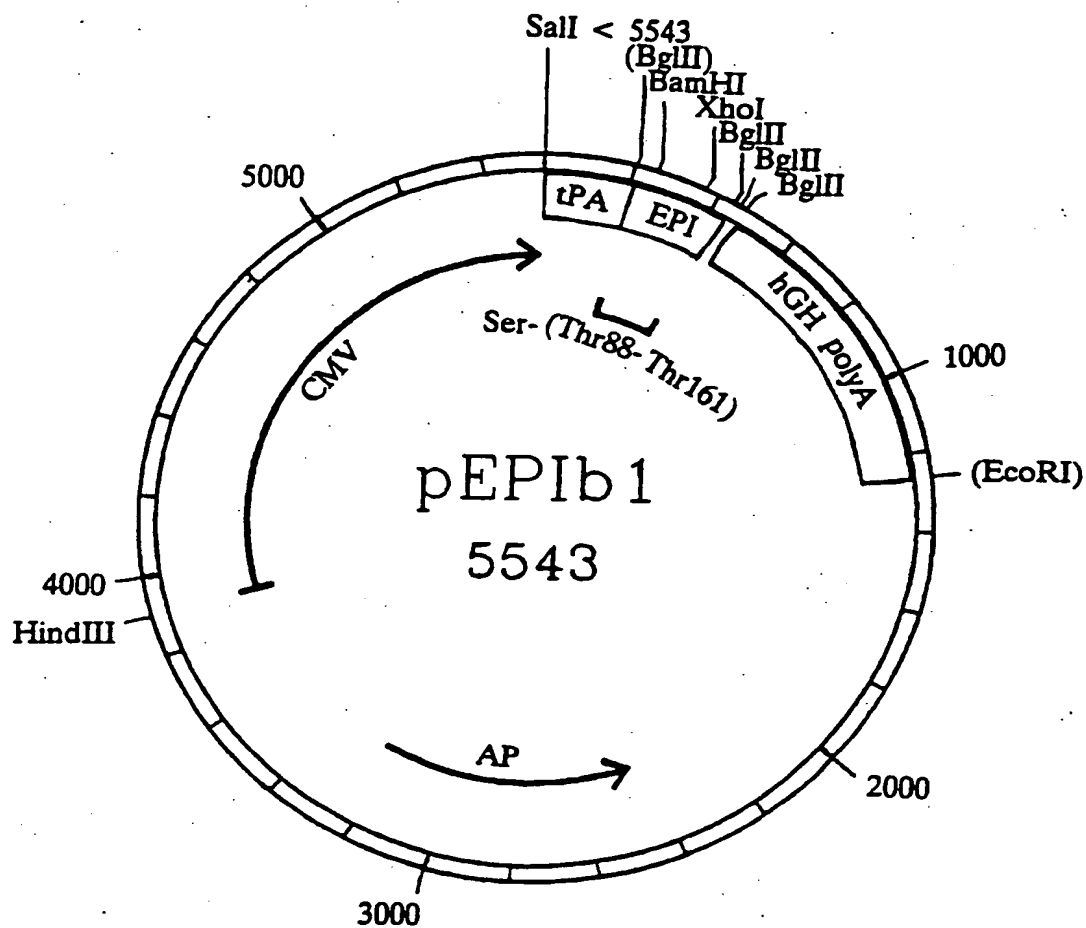


Fig. 5

REPLACEMENT SHEET

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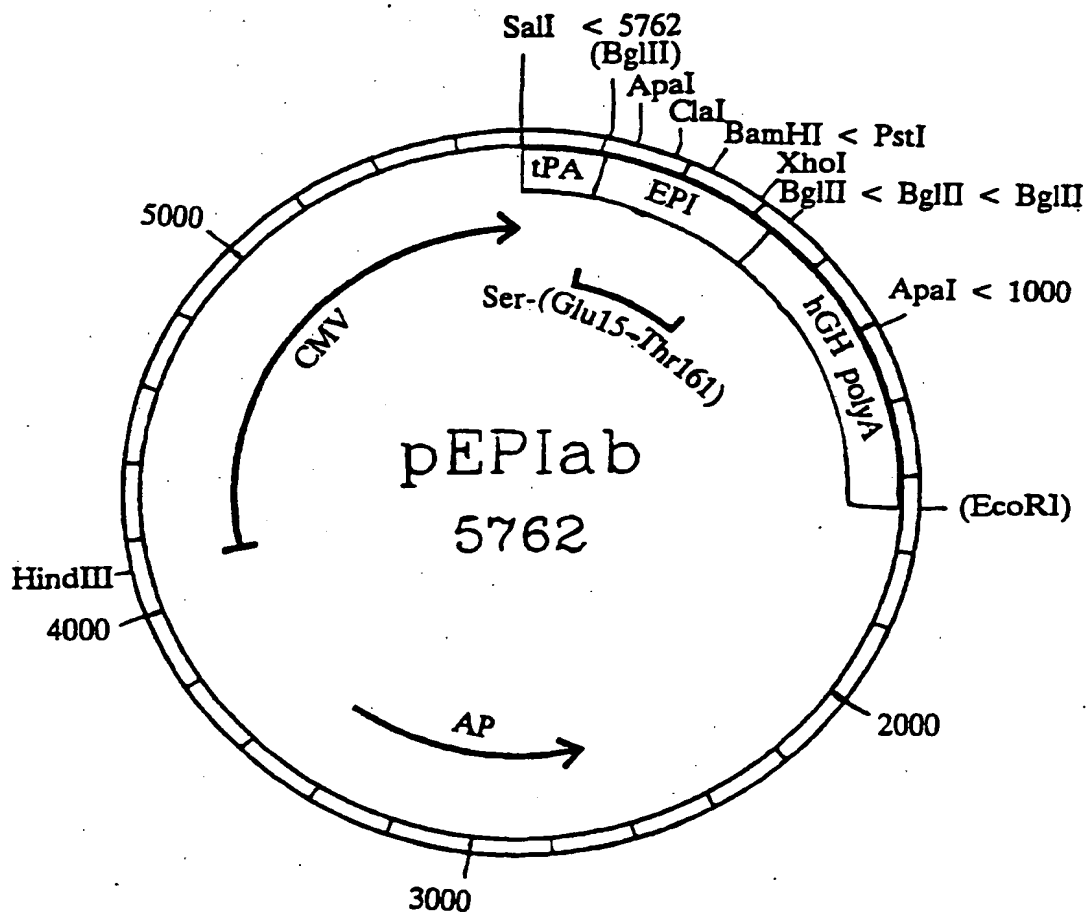


Fig. 6

REPLACEMENT SHEET

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| | |
|--|-----|
| GTCGACAGAG CTGAGATCCT ACAGGAGTCC AGGGCTGGAG AGAAAACCTC | 50 |
| TGCGAGGAAA GGGAAGGAGC AAGCCGTGAA TTAAAGGGAC GCTGTGAAGC | 100 |
| AATC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG | 143 |
| Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu | |
| 1 5 10 | |
| CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT | 185 |
| Leu Cys Gly Ala Val Phe Val Ser Pro Ser Gln Glu Ile His | |
| 15 20 25 | |
| GCC CGA TTC AGA AGA GGA GCC AGA TCA ACA CTG CAG CAA GAA | 227 |
| Ala Arg Phe Arg Arg Gly Ala Arg Ser Thr Leu Gln Gln Glu | |
| 30 35 40 | |
| AAG CCA GAT TTC TGC TTT TTG GAA GAG GAT CCT GGA ATA TGT | 269 |
| Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro Gly Ile Cys | |
| 45 50 55 | |
| CGA GGT TAT ATT ACC AGG TAT TTT TAT AAC AAT CAG ACA AAA | 311 |
| Arg Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn Asn Gln Thr Lys | |
| 60 65 | |
| CAG TGT GAA AGG TTC AAG TAT GGT GGA TGC CTG GGC AAT ATG | 353 |
| Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Leu Gly Asn Met | |
| 70 75 80 | |
| AAC AAT TTT GAG ACA CTC GAG GAA TGC AAG AAC ATT TGT GAA | 395 |
| Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu | |
| 85 90 95 | |
| GAT GGT CCG AAT GGT TTC CAG GTG GAT AAT TAT GGT ACC TGA | 437 |
| Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr Gly Thr End | |
| 100 105 110 | |
| BglIII BglIII BglIII | |
| AGATCTGAAT TCTGAAGATC TAGGCCTATG AAGATCT | 474 |

Fig. 7

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| | | | | | | | | | | | | | | |
|--|------------|------------|------------|------------|--|-----|-----|--|----|--|--|--|-----|-----|
| Sali | | | | | | | | | | | | | | |
| GTCTGACAGAG | CTGAGATCCT | ACAGGAGTCC | AGGGCTGGAG | AGAAAACCTC | | | | | | | | | | 50 |
| TGCCGAGGAAA | GGAAGGAGC | AAGCCGTGAA | TTTAAGGGAC | GCTGTGAAGC | | | | | | | | | | 100 |
| AATC ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG | | | | | | | | | | | | | | 143 |
| Met Asp Ala Met Lys Arg Gly Leu Cys Cys Val Leu Leu | | | | | | | | | | | | | | |
| 1 | | | 5 | | | | | | | | | | 10 | |
| CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT | | | | | | | | | | | | | | 185 |
| Leu Cys Gly Ala Val Phe Val Ser Pro Ser Gln Glu Ile His | | | | | | | | | | | | | | |
| 15 | | | 20 | | | | | | | | | | 25 | |
| SacI | | | | | | | | | | | | | | |
| GCC CGA TTC AGA AGA GGA GCC AGA TCA GAG CTC CCA CCA CTG | | | | | | | | | | | | | | 227 |
| Ala Arg Phe Arg Arg Gly Ala Arg Ser Glu Leu Pro Pro Leu | | | | | | | | | | | | | | |
| 30 | | | | | | 35 | | | | | | | 40 | |
| ApaI | | | | | | | | | | | | | | |
| AAA CTT ATG CAT TCA TTT TGT GCA TTC AAG GCG GAT GAT GGG | | | | | | | | | | | | | | 269 |
| Lys Leu Met His Ser Phe Cys Ala Phe Lys Ala Asp Asp Gly | | | | | | | | | | | | | | |
| 45 | | | | | | | 50 | | | | | | 55 | |
| CCC TGT AAA GCA ATC ATG AAA AGA TTT TTC TTC AAT ATT TTC | | | | | | | | | | | | | | 311 |
| Pro Cys Lys Ala Ile Met Lys Arg Phe Phe Phe Asn Ile Phe | | | | | | | | | | | | | | |
| 60 | | | | | | | 65 | | | | | | | |
| ACT CGA CAG TGC GAA GAA TTT ATA TAT GGG GGA TGT GAA GGA | | | | | | | | | | | | | | 353 |
| Thr Arg Gln Cys Glu Glu Phe Ile Tyr Gly Gly Cys Glu Gly | | | | | | | | | | | | | | |
| 70 | | | | 75 | | | | | 80 | | | | | |
| ClaI | | | | | | | | | | | | | | |
| AAT CAG AAT CGA TTT GAA AGT CTG GAA GAG TGC AAA AAA ATG | | | | | | | | | | | | | | 395 |
| Asn Gln Asn Arg Phe Glu Ser Leu Glu Glu Cys Lys Lys Met | | | | | | | | | | | | | | |
| 85 | | | | | | 90 | | | | | | | 95 | |
| PstI | | | | | | | | | | | | | | |
| TGT ACA AGA GAT AAT GCA AAC AGG ATT ATA AAG ACA ACA CTG | | | | | | | | | | | | | | 437 |
| Cys Thr Arg Asp Asn Ala Asn Arg Ile Ile Lys Thr Thr Leu | | | | | | | | | | | | | | |
| 100 | | | | | | 105 | | | | | | | 110 | |
| BamHI | | | | | | | | | | | | | | |
| CAG CAA GAA AAG CCA GAT TTC TGC TTT TTG GAA GAG GAT CCT | | | | | | | | | | | | | | 479 |
| Gln Gln Glu Lys Pro Asp Phe Cys Phe Leu Glu Glu Asp Pro | | | | | | | | | | | | | | |
| 115 | | | | | | | 120 | | | | | | 125 | |
| GGA ATA TGT CGA GGT TAT ATT ACC AGG TAT TTT TAT AAC AAT | | | | | | | | | | | | | | 521 |
| Gly Ile Cys Arg Gly Tyr Ile Thr Arg Tyr Phe Tyr Asn Asn | | | | | | | | | | | | | | |
| 130 | | | | | | | 135 | | | | | | | |

Fig. 8

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| | |
|---|-----|
| CAG ACA AAA CAG TGT GAA AGG TTC AAG TAT GGT GGA TGC CTG | 563 |
| Gln Thr Lys Gln Cys Glu Arg Phe Lys Tyr Gly Gly Cys Leu | |
| 140 145 150 | |
| XhoI | |
| GGC AAT ATG AAC AAT TTT GAG ACA CTC GAG GAA TGC AAG AAC | 605 |
| Gly Asn Met Asn Asn Phe Glu Thr Leu Glu Glu Cys Lys Asn | |
| 155 160 165 | |
| ATT TGT GAA GAT GGT CCG AAT GGT TTC CAG GTG GAT AAT TAT | 647 |
| Ile Cys Glu Asp Gly Pro Asn Gly Phe Gln Val Asp Asn Tyr | |
| 170 175 180 | |
| KpnI BglIII BglIII BglIII | |
| GGT ACC TGA AGATCTGAAT TCTGAAGATC TAGGCCTATG AAGATCT | 693 |
| Gly Thr End | |

Fig. 8 cont.

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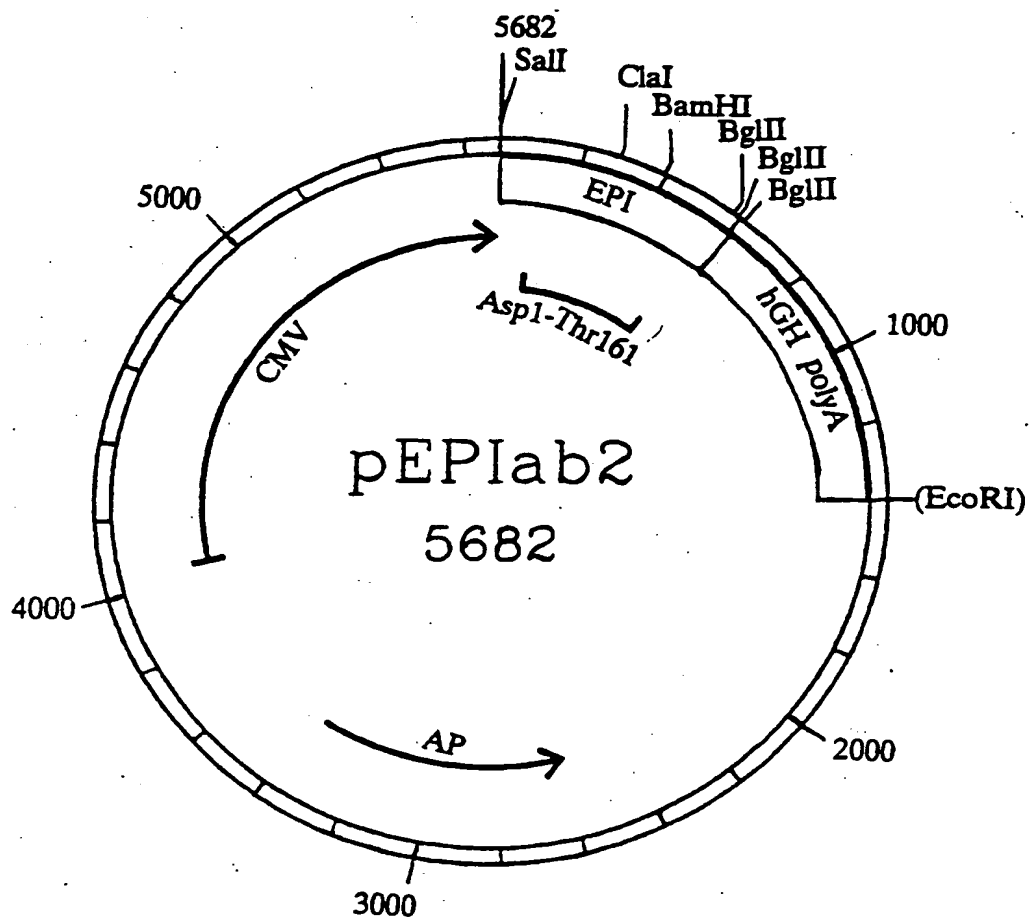


Fig. 9

REPLACEMENT SHEET

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| | | | | | | | | | | | | | | |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Sali | | | | | | | | | | | | | | |
| GTCGACC | ATG | ATT | TAC | ACA | ATG | AAG | AAA | GTA | CAT | GCA | CTT | TGG | | 43 |
| | Met | Ile | Tyr | Thr | Met | Lys | Lys | Val | His | Ala | Leu | Trp | | |
| | 1 | | | | 5 | | | | | 10 | | | | |
| GCT | AGC | GTA | TGC | CTG | CTG | CTT | AAT | CTT | GCC | CCT | GCC | CCT | CTT | 85 |
| Ala | Ser | Val | Cys | Leu | Leu | Leu | Asn | Leu | Ala | Pro | Ala | Pro | Leu | |
| | 15 | | | | | 20 | | | | 25 | | | | |
| AAT | CGT | GAT | TCT | GAG | GAA | GAT | GAA | GAA | CAC | ACA | ATT | ATC | ACA | 127 |
| Asn | Ala | Asp | Ser | Glu | Glu | Asp | Glu | Glu | His | Thr | Ile | Ile | Thr | |
| | | 30 | | | | | 35 | | | | | 40 | | |
| GAT | ACG | GAG | CTC | CCA | CCA | CTG | AAA | CTT | ATG | CAT | TCA | TTT | TGT | 169 |
| Asp | Thr | Glu | Leu | Pro | Pro | Leu | Lys | Leu | Met | His | Ser | Phe | Cys | |
| | | | 45 | | | | | 50 | | | | | | |
| GCA | TTC | AAG | GCG | GAT | GAT | GGG | CCC | TGT | AAA | GCA | ATC | ATG | AAA | 211 |
| Ala | Phe | Lys | Ala | Asp | Asp | Gly | Pro | Cys | Lys | GCA | Ala | Ile | Met | |
| 55 | | | | 60 | | | | | 65 | | | | | |
| AGA | TTT | TTC | TTC | AAT | ATT | TTC | ACT | CGA | CAG | TGC | GAA | GAA | TTT | 253 |
| Arg | Phe | Phe | Phe | Asn | Ile | Phe | Thr | Arg | Gln | Cys | Glu | Glu | Phe | |
| | 70 | | | | 75 | | | | | 80 | | | | |
| ClaI | | | | | | | | | | | | | | |
| ATA | TAT | GGG | GGA | TGT | GAA | GGA | AAT | CAG | AAT | CGA | TTT | GAA | AGT | 295 |
| Ile | Tyr | Gly | Gly | Cys | Glu | Gly | Asn | Gln | Asn | Arg | Phe | Glu | Ser | |
| | 85 | | | | | 90 | | | | | 95 | | | |
| CTG | GAA | GAG | TGC | AAA | AAA | ATG | TGT | ACA | AGA | GAT | AAT | GCA | AAC | 337 |
| Leu | Glu | Glu | Cys | Lys | Lys | Met | Cys | Thr | Arg | Asp | Asn | Ala | Asn | |
| | | | 100 | | | | | 105 | | | | | | |
| AGG | ATT | ATA | AAG | ACA | ACA | CTG | CAG | CAA | GAA | AAG | CCA | GAT | TTC | 379 |
| Arg | Ile | Ile | Lys | Thr | Thr | Leu | Gln | Gln | Glu | Lys | Pro | Asp | Phe | |
| 110 | | | | 115 | | | | | 120 | | | | | |
| BamHI | | | | | | | | | | | | | | |
| TGC | TTT | TTG | GAA | GAG | GAT | CCT | GGA | ATA | TGT | CGA | GGT | TAT | ATT | 421 |
| Cys | Phe | Leu | Glu | Glu | Asp | Pro | Gly | Ile | Cys | Arg | Gly | Tyr | Ile | |
| | 125 | | | | | 130 | | | | | 135 | | | |
| ACC | AGG | TAT | TTT | TAT | AAC | AAT | CAG | ACA | AAA | CAG | TGT | GAA | AGG | 463 |
| Thr | Arg | Tyr | Phe | Tyr | Asn | Asn | Gln | Thr | Lys | Gln | Cys | Glu | Arg | |
| | | 140 | | | | 145 | | | | | 150 | | | |
| TTC | AAG | TAT | GGT | GGA | TGC | CTG | GGC | AAT | ATG | AAC | AAT | TTT | GAG | 505 |
| Phe | Lys | Tyr | Gly | Gly | Cys | Leu | Gly | Asn | Met | Asn | Asn | Phe | Glu | |
| | | | 155 | | | | 160 | | | | | | 165 | |

Fig. 10

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| | |
|---|-----|
| AGA CTC GAG GAA TGC AAG AAC ATT TGT GAA GAT GGT CCG AAT | 547 |
| Thr Leu Glu Glu Cys Lys Asn Ile Cys Glu Asp Gly Pro Asn | |
| 170 175 | |

| | | |
|---|------------|-----|
| GGT TTC CAG GTG GAT AAT TAT GGT ACC TGA | BglII | 587 |
| Gly Phe Gln Val Asp Asn Tyr Gly Thr End | AGATCTGAAT | |
| 180 185 | | |

| | | |
|-------------------------------|-------|-----|
| BglII | BglII | |
| TCTGAAGATC TAGGCCTATG AAGATCT | | 614 |

Fig. 10 cont.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/DK 90/00212

| | | |
|--|--|---|
| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 07 K 13/00, C 12 N 15/15, A 61 K 37/64 | | |
| II. FIELDS SEARCHED Minimum Documentation Searched ⁷ | | |
| Classification System | Classification Symbols | |
| IPC5 | C 07 K; C 12 N; A 61K | |
| Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched ⁸ | | |
| SE,DK,FI,NO classes as above | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ | | |
| Category ¹⁰ | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ |
| X | Nature, Vol. 338, 1989 J. Girard et al.: "Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor", see especially p. 518 col. 2 lines 11-12, p. 520 col 1 lines 1-8 | 1-3,14-16 |
| Y | -- | 17 |
| Y | EP, A2, 0318451 (MONSANTO COMPANY) 31 May 1989, see the whole document | 17 |
| A | EP, A2, 0300988 (WASHINGTON UNIVERSITY) 25 January 1989, see the whole document | 1-18 |
| <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> | | |
| IV. CERTIFICATION | | |
| Date of the Actual Completion of the International Search | | Date of Mailing of this International Search Report |
| 12th November 1990 | | 1990 -11- 19 |
| International Searching Authority | | Signature of Authorized Officer |
| SWEDISH PATENT OFFICE | | Mikael G:son Bergstrand |

Form PCT/ISA/210 (second sheet) (January 1985)

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|---|----------------------|
| Category | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
| A | Thrombosis research, Vol. 48, 1987 B.J. Warr-Cramer et al.: "Partial Purification and characterization of extrinsic ", see page 11 - page 22 -- | 1-18 |
| A | Proc. Natl. Acad. Sci., Vol. 84, 1987 George J. Broze, Jr. et al.: "Isolation of the tissue factor inhibitor produced by HepG2 hepatoma cells ", see page 1886 - page 1890 ----- | 1-18 |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claim numbers.....1....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claim states that the EPI analogue has a low heparin binding capacity, without describing anything about the structure of the analogue and hence, the claim is considered not to be clear and concise, c.f. PCT article 6.

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 90/00212**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 90-09-27. The Swedish Patent Office is in no way liable for those particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|--------------------------------|----------------------|
| EP-A2- 0318451 | 89-05-31 | AU-D- 1928788 JP-A- 1165383 | 89-05-25 89-06-29 |
| EP-A2- 0300988 | 89-01-25 | AU-D- 1928688 JP-A- 1047799 | 89-02-02 89-02-22 |